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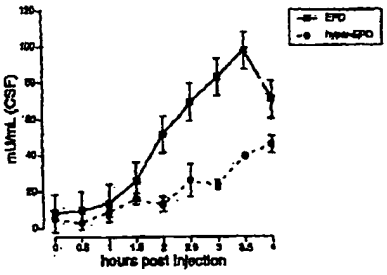
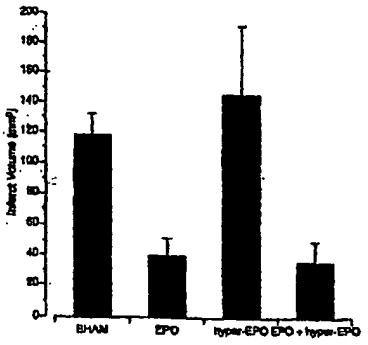
(54) Title: LONG ACTING ERYTHROPOIETINS THAT MAINTAIN TISSUE PROTECTIVE ACTIVITY OF ENDOGENOUS ERYTHROPOIETIN





(57) Abstract: Methods for increasing the hematocrit of an individual while maintaining the tissue protective activities of endogenous through the administration of a pharmaceutical compound containing chemically modified long acting erythropoietin. Also disclosed are the new chemically modified long acting erythropoietins, methods of producing the chemically modified long acting erythropoietins, and compositions comprising the chemically modified long acting erythropoietins.

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LONG ACTING ERYTHROPOIETINS THAT MAINTAIN TISSUE PROTECTIVE ACTIVITY OF ENDOGENOUS ERYTHROPOIETIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/409,020, filed on September 9, 2002, which is incorporated in its entirety by reference herein.

FIELD OF THE INVENTION

The present invention relates to long acting erythropoietins that advantageously maintain tissue protective capabilities after modification. In particular, the present invention relates to long acting erythropoietins that are chemically modified in a way that increases the serum half-life but also maintains the tissue protective function of the native protein *in vivo*. The present invention also relates to the treatment of anemia and anemia related diseases with the long acting erythropoietins of the present invention. Finally, the present invention is directed to assays useful in the determination of whether an erythropoietin exhibits tissue protective capabilities.

BACKGROUND OF THE INVENTION

Naturally occurring or endogenous erythropoietin (EPO) is a glycoprotein hormone produced mainly in the liver. Endogeneous EPO includes 165 amino acids and has a molecular weight (in humans) of about 30,000 to about 34,000 daltons. The glycosyl residues in EPO, which consist of three N-linked and one O-linked oligosaccharide chains, are responsible for about 40 percent of the protein's total weight. The N-linked oligosaccharide chains are bonded to amide nitrogens of asparagine at positions 24, 38 and 83, while the O-linked oligosaccharide chain is bonded to the oxygen at the serine residue located at position 126. The EPO protein may occur in three forms: α, β, and asialo. The α and β forms have the same potency, biological activity, and molecular weight, but differ slightly in the carbohydrate components, while the asialo form is an α or β form with the terminal sialic acid (carbohydrate) removed.

Until recently, the principle function of endogeneous EPO is to act in concert with other growth factors to stimulate the proliferation and maturation of responsive bone marrow erythroid precursor cells and maintain an individual's hematocrit (percent of whole blood that contains red blood cells). The process of producing the red blood cells is called erythropoiesis, which is a

Recent studies also have suggested that systemically administered EPO may cross the intact blood brain barrier because the capillaries forming the blood brain barrier also express the EPO receptor. As such, an anatomical basis for receptor-mediated transcytosis is provided from the peripheral circulation into the brain.

Recombinant EPO (epoetin alfa), which has been commercially available under tradenames PROCRIT® (from Ortho Biotech Inc., Raritan, NJ), and EPOGEN® (from Amgen, Inc., Thousand Oaks, CA), has been used to treat anemia resulting from end stage renal disease, to treat HIV-infected patients when used in concert with AZT (zidovudine) therapy, and to counterbalance the effects of chemotherapy. While the therapeutic effects of recombinant EPO are numerous, to date the principal application of recombinant EPO has been to address chronic anemia. In this regard, recombinant EPO is typically administered in an initial dose of between 50-150 units/kg three times per week for about six to eight weeks either by an intravenous or subcutaneous injection in order to restore the suggested hematocrit range within the patient. After the patient achieves a desired hematocrit level, such as an amount falling within from about 30 percent to about 36 percent, that level may be sustained by maintenance EPO therapy in the absence of iron deficiency and concurrent illnesses. While dosage requirements may vary according to the patient's individual needs, typically maintenance dosages may be administered about three times a week (less if larger doses are provided).

The dosage amount and frequency of the administration of recombinant EPO is determined in part upon the half-life of the molecule, which may be limited when the molecule is in vivo. For example, intravenously administered EPOGEN® is reportedly eliminated at a rate consistent with first order kinetics with a circulating half-life ranging from approximately 4 to 13 hours in adult and pediatric patients with CRF. Thus, in order to be therapeutically effective, the dosage amount and frequency of dosing must be tailored to account for the relatively short half-life of the recombinant EPO.

Additionally, because recombinant EPO is administered either by an intravenous or subcutaneous injection, a nurse or physician often is required to administer recombinant EPO to a patient. This presents an additional inconvenience to a patient, and is yet another reason why it may be desirable to extend the half-life of the molecule. As such, efforts to increase the half-life of recombinant EPO have gained research attention in the past decade based on the premise that an extended half-life would decrease dosage requirements while still providing the same or improved therapeutic benefits.

BRIEF SUMMARY OF THE INVENTION

The present invention is directed to a method for regulating the hematocrit level in humans including the steps of providing an erythropoietin product having a longer serum half-life than recombinant human erythropoietin (rhuEPO) and including tissue protective functionality and administering a therapeutically effective amount of the erythropoietin product. In one embodiment, the step of providing an erythropoietin product further includes the step of modifying recombinant erythropoietin with at least one chemical modification to at least one of the N-linked oligosaccharide chains or the O-linked oligosaccharide chain, wherein the chemical modification includes oxidation, sulfation, phosphorylation, PEGylation, or a combination thereof.

In adddition, the step of administering a therapeutically effective amount of the erythropoietin product may include administering the erythropoietin product at a lower molar amount than rhuEPO to obtain a comparable target hematocrit.

In one embodiment, the serum half-life is at least about 20 percent longer than the serum half-life of rhuEPO. In another embodiment, the serum half-life is at least about 40 percent longer than the serum half-life of rhuEPO.

The present invention is also directed to a man-made erthyropoietin product including at least one erythropoietin derivative, wherein at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain has at least one chemical modification as a result of oxidation, sulfation, phosphorylation, PEGylation, or mixtures thereof, and wherein the erythropoietin product has a longer serum half-life than rhuEPO. The erythropoietin product preferably has tissue protective functionality.

In one embodiment, the at least one chemical modification includes oxidation of at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain to provide at least one additional acid residue. For example, the at least one chemical modification may include sulfation of at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain to provide an increased negative charge on the EPO product. In another embodiment, the at least one chemical modification includes phosphorylation of at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain to provide an increased negative charge on the EPO product. In still another embodiment, the at least one chemical modification includes addition of at least one polyethylene glycol chain to at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain.

obtain a comparable target hematocrit, wherein the erythropoietin product has tissue protective functionality.

In this aspect of the invention, the erythropoietin product preferably has a longer serum half-life than rhuEPO. In one embodiment, the serum half-life is at least about 20 percent longer than the serum half-life of rhuEPO. In another embodiment, the serum half-life is at least about 40 percent longer than the serum half-life of rhuEPO.

The present invention further relates to a pharmaceutical composition including: a therapeutically effective amount of at least one erythropoietin derivative, wherein at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain has at least one chemical modification as a result of oxidation, sulfation, phosphorylation, PEGylation, or mixtures thereof, wherein the at least one erythropoietin derivative has a longer serum half-life than recombinant erythropoietin and has tissue protective functionality. In one embodiment, the pharmaceutical composition further includes at least one pharmaceutically acceptable carrier. The at least one pharmaceutically acceptable carrier may include at least one diluent, adjuvant, excipient, vehicle, or mixtures thereof.

In another embodiment, the pharmaceutical composition further includes at least one wetting agent, emulsifying agent, pH buffering agent, or a combination thereof. In yet another embodiment, the pharmaceutical composition further includes at least one tissue protective cytokine.

BRIEF DESCRIPTION OF THE FIGURES

Further features and advantages of the invention can be ascertained from the following detailed description that is provided in connection with the drawings described below:

FIG. 1A is a comparison of the effectiveness of various forms of EPO in protecting against cell death triggered by exposure to trimethyl tin; and

FIG. 1B is a comparison of the effectiveness of various forms of EPO in protecting against cell death triggered by exposure to trimethyl tin.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the use of EPO molecules having an extended serum half-life (long acting) that are chemically modified with carbohydrate chains so that the functionality of endogenous EPO is maintained. As discussed in the background, efforts to extend the half-life of EPO have generally been focused on adding extra carbohydrate chains to the EPO molecule to

acting EPO of the present invention results from the addition of polyethylene glycol to the carbohydrate chain of EPO. Any combination of the foregoing modifications is also contemplated by the present invention. And, as mentioned above, the present invention also embraces compositions, including pharmaceutical compositions, which include one or more of the aforementioned long acting EPO molecules.

The long acting EPO molecules of the present invention are contemplated for inclusion in pharmaceutical compositions for treating anemia and related diseases, especially those with complications resulting from illnesses such as, but limited to, acute renal failure, sepsis, HIV, chemotherapy, and the like.

The present invention is also directed to methods for treating anemia and related diseases, as well as kits used for the treatment procedure. As used herein, the term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. The present invention contemplates the use of the long acting EPOs for chronic administration, acute treatment, and/or intermittent administration. For the purposes of this disclosure, "chronic administration" refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time and "intermittent administration" is treatment that is not consecutively done without interruption, but rather is cyclic in nature.

The long acting EPOs of the present invention, and the uses thereof, are applicable for any mammal. As used herein, the term "mammal" refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, etc. Preferably, the mammal is human. The administration of the long acting EPOs of the present invention include, but is not limited to, oral, intravenous, intranasal, topical, intraluminal, inhalation or parenteral administration, the latter including intravenous, intraarterial, subcutaneous, intramuscular, intraperitoneal, submucosal, intradermal, and combinations thereof.

The present invention further relates to the use of long acting EPO molecules of the present invention as a carrier for other molecules into areas of the body that have EPO receptors. For example, because certain molecules have poor penetration across the blood brain barrier, linking these molecules to the long acting EPOs of the present invention provides a safe and effective

Tissue Protective Capability

To evaluate the possibility that extra carbohydrate chains may affect the functionality of the glycoprotein, the present inventors studied forms of EPO analogs that have five N-linked carbohydrate chains (compared to the 3 N-linked carbohydrate chains of recombinant EPO). In particular, the inventors used an EPO analog, wherein the analog has extra glycosylation site at the 32 amino acid, which results in about a 3-fold longer half-life than recombinant EPO (epoetin alfa).

Although this EPO analog appeared within the cerebral spinal fluid after systemic injection (Figures 1A), it was surprisingly not tissue protective when evaluated in a subsequent P19 *in vitro* assay (Figure 1B). This lack of tissue protective activity was unexpected. In addition, the lack of tissue protective activity may produce complications when used for treatment in anemia patients if those patients have other conditions requiring the tissue protective capability. For example, if the non-tissue protective EPO analog competes with tissue protective endogenous EPO for the receptor that triggers the tissue protective response, the extent of injury resulting from a trauma may actually be exacerbated due to the use of such EPO analogs. In fact, if a patient on such an EPO analog suffered from a stroke, the infarct volume resulting from the stroke may actually be greater than in an individual not treated with an EPO analog.

While not wishing to be bound to any particular theory, this finding suggests that at least one additional version of an EPO receptor functionally exists in neuronal tissues for which signaling differs from that of erythrocyte precursors, and there is a risk that certain EPO analogs may antagonize endogenous EPO's ability to bind to this version of the receptor. The distinctly different biological activities between endogenous EPO and these EPO analogs suggest that receptor signaling occurs via functionally different EPO receptors responding to different domains of the EPO molecule. In fact, while the EPO receptor gene protein sequence has been reported to be identical to that expressed by the erythroid precursors, the binding affinity of the neuronal-type receptor for EPO *in vitro* is much lower than the EPO receptor of the proerythrocyte. *See, e.g.*, Masuda, S., et al., *J Biol Chem*, 268, 11208-16 (1993). Presumably, these differences arise from accessory proteins and may indicate that different signaling pathways are employed than those activated in the erythrocyte maturation program. Interestingly, this difference in affinity is not modified by complete deglycosylation of EPO, a result that is not unexpected if the neurally-active binding occurs in the normally non-glycosylated AB loop region of EPO. *Id.* In addition, EPO produced by astrocytes (which is presumably the same product produced by other cells such as neurons) is also a smaller version than that produced by the kidney. Masuda, S., *J. Bio Chem*, 269, 19488-93 (1994). The

barrier, as well as other barriers with capillaries expressing the EPO receptor.

In sum, because the EPO analogs of the prior art have been shown to maintain erythropoietic activity at the sacrifice of at least some of the functionality of endogenous EPO, there exists a need in the art for a long acting EPO that maintains all of the known functionality of endogenous EPO. Advantageously, the present invention is directed to a long acting EPO of the present invention that not only increases the serum half-life as compared to recombinant EPO, but also maintains the functionality of endogenous EPO, *i.e.*, the tissue protective functionality and the transcytosis capability. Various methods of modifying EPO to provide such a beneficial protein are provided in the next section.

Modification of Native EPO

The long acting EPOs of the present invention may be formed in a variety of ways. In general, the long acting EPOs may be generated by chemically modifying the carbohydrate (sugar) chains attached to the EPO. As used herein, the term "carbohydrate chains" refer to the N-linked and O-linked oligosaccharide chains found in endogenous EPO, the additional N-linked and O-linked oligosaccharide chains found in EPO analogs, and any other carbohydrate chains, specifically sugar chains, attached to EPO.

In one embodiment, endogenous or recombinant EPO is used for modification so as to prevent any interference with the tissue protective capabilities of endogenous EPO. In addition, EPO analogs are contemplated for modification according to the present invention providing the additional glycosylation sites are not located near the O'Brien peptide, *i.e.*, the 30-47 amino acid sequence. As used herein, the term "EPO analogs" refers to modified EPO molecules that have at least one additional N-linked carbohydrate chain and/or at least one additional O-linked carbohydrate chain. In one embodiment, an EPO analog used for modification does not include any additional glycosylation sites within about 5 amino acids of the O'Brien peptide. In another embodiment, the EPO analog does not include any additional glycosylation sites within about 3 amino acids of the O'Brien peptide. In still another embodiment, the EPO analog does not include any additional glycosylation sites within the O'Brien peptide.

An EPO analog may also be used for modification according to the present invention provided that the analog is reviewed in three-dimensional space and it is confirmed that none of the additional carbohydrate chains do not block the O'Brien peptide or cause a loss of tissue protective functionality. In another aspect, an EPO analog is contemplated for use in modification according to

periodate, and sugar oxidases, such as galactose oxidase. In addition, skilled artisans would be aware of suitable reagents for transforming the aldehydes, such as Quantitative Benedict Solution (commercially available from Fisher). In one embodiment, the sugar molecules are oxidized with sodium periodate and further treated with Quantitative Benedict Solution (Fisher) to convert the aldehydes into acids.

In another embodiment, an EPO isomer, one having about 0-13 sialic acid residues, or an EPO analog, having at least one carbohydrate chain that lacks a sialic acid residue, is subjected to oxidation using galactose oxidase. An asialo form of EPO may be used according to this aspect of the invention, *i.e.*, an α or β form of EPO with the terminal carbohydrate (sialic acid) removed. Preferably, asialoerythropoietin is used. Once oxidized, the EPO is subjected to another oxidative agent, such as Quantitative Benedict Solution, to transform the aldehydes into acids.

In yet another embodiment, a ruthenium tetroxide system may be used to generate the acids on the sugar chain. Given that these modifications involve the galactose chain even if the acids involved in these transformations are stripped from the EPO molecule, the molecule should be able to evade removal by the liver since a galactose chain, the component that the liver screens for, will not be exposed.

Increasing the Negative Charge

In another aspect of the present invention, a long acting EPO of the present invention is formed by adding sulfates and/or phosphates to the EPO molecule, which will increase the negative charge of the molecule and thereby increase the half-life of the molecule. In other words, the negative charge of the EPO molecule may be increased by sulfation, which involves the transfer of a sulfuryl group from a sulfate donor, including protein, glycolipids, glycosaminoglycans and steroids. And, the negative charge may also be increased by introducing a phosphoric group into a carbohydrate.

One suitable method for sulfation of insulin is discussed in S. Pongor *et al.*, Preparation of High-Potency, Non-aggregating Insulins Using a Novel Sulfation Procedure, Diabetes, Vol. 32, No. 12, December 1983. For example, insulin sulfation was carried out in an organic solvent, such as dimethylformamide (DMF), in the presence of condensing agents, such as N,N'-dicyclohexyl carbodiimide (DCC), and a sulfate donor. The degree of sulfation can be controlled over an eightfold range by varying the amount of condensing agent. Although conventionally prepared sulfated insulin resulted in major insulin bioactivity loss, the bioactivity of the sulfated insulin made with the Ponger

electrophilically activated PEG, such as mPEG-succinimidyl propionate (mPEG-SPA) or mPEG-succinimidyl butanoate (mPEG-SBA), both of which are commercially available from Nektar Therapeutics of Birmingham, Alabama. In yet another embodiment, the PEG is a methoxy PEG-hydrazide.

In one embodiment, the addition of the at least one PEG is achieved via oxidation with periodate (as disclosed above), followed by the use of cyanoborohydride and an amino PEG. For example, EPO in solution may be first oxidized with a periodate, *e.g.*, sodium periodate, for a predetermined period of time at room temperature, which produces aldehydes in the carbohydrate chains. A suitable periodate is sodium meta-periodate, which is commercially available from Sigma. The periodate may then be removed by buffer exchange, at which time the oxidized sialic acid groups on N-linked oligosaccharide groups of EPO may be subjected to at least one amino PEG in the presence of cyanoborohydride. Suitable PEGs for use include, but are not limited to, methoxy-PEG-hydrazides, which are commercially available from Nektar Therapeutics.

In another embodiment, the addition of the at least one PEG is performed by the attachment of PEG groups to terminal galactose residues after oxidation with galactose oxidase. For example, an asialo form of EPO (having exposed terminal galactose residues) in buffer is first subjected to galactose oxidase (commercially available from Sigma) to generate aldehydes in the carbohydrate chains. The buffer may then be removed by buffer exchange, at which time the oxidized galactose residues may be subjected to at least one amino PEG in the presence of cyanoborohydride.

The methods provided above are not intended to be limiting as these or other methods may be used to prepare the compounds of the invention. For example, a skilled artisan would recognize the applicability of these chemical modifications to creating long acting versions of other EPO derivatives such as the tissue protective cytokines disclosed in International Publication No. WO/02053580 and U.S. Patent Publication Nos. 2002/0086816 and 2003/0072737, which are incorporated by reference herein in their entirety.

Production of the EPO Molecules

A variety of host-expression vector systems may be utilized to produce the long acting EPO and EPO-related molecules of the invention. Such host-expression systems represent vehicles by which the long acting EPOs of interest may be produced and subsequently purified, but also represent cells that may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the modified erythropoietin gene product *in situ*. These include but are not limited to,

Alternatively, the expression characteristic of an endogenous EPO mutein gene within a cell line or microorganism may be modified by inserting a heterologous DNA regulatory element into the genome of a stable cell line or cloned microorganism such that the inserted regulatory element is operatively linked with the endogenous erythropoietin mutein gene. For example, an endogenous EPO mutein gene that is normally "transcriptionally silent", *i.e.*, an EPO gene that is normally not expressed, or is expressed only at very low levels in a cell line, may be activated by inserting a regulatory element that is capable of promoting the expression of an expressed gene product in that cell line or microorganism. Alternatively, a transcriptionally silent, endogenous EPO gene may be activated by insertion of a promiscuous regulatory element that works across cell types.

A heterologous regulatory element may be inserted into a stable cell line or cloned microorganism, such it is operatively linked with an endogenous erythropoietin gene, using techniques, such as targeted homologous recombination, which are well known to those of skill in the art, and also described French Patent No. 2646438, U.S. Patent Nos. 4,215,051 and 5,578,461, and International Publication Nos. WO93/09222 and WO91/06667, the entire disclosures of which are incorporated by reference herein.

Pharmaceutical Compositions

The present invention also relates to pharmaceutical compositions including the long acting EPO molecules of the present invention. Because the long acting EPOs of the present invention advantageously have erythropoietic activity, as well as tissue protective capability and transcytosis capability, they are contemplated for treatment of anemia and related diseases in individuals also at risk for various tissue injuries, such as stroke and myocardial infarction. In addition, the long acting EPOs of the present invention are contemplated for treatment of anemia and related diseases in individuals also experiencing deteroriation of mental faculties, such as Alzheimer's, Parkinson's and the like. Furthermore, the long acting EPOs of the present invention are contemplated for the treatment of anemia in individuals subject to conditions resulting from the normal aging process, *e.g.*, balance problems leading to falling, easy bruising, and the like. Moreover, the present invention relates to the use of the long acting EPOs of the present invention as carriers for other molecules that have poor penetration across barriers with capillaries having EPO receptors.

For example, any of the long acting EPOs discussed above may be included in pharmaceutical compositions of the invention. In addition, various EPO analogs may be included in pharmaceutical compositions of the invention in a blend with at least one tissue protective cytokine, which will be

Composition Including Long Acting EPO

As briefly mentioned above, any of the long acting EPOs of the present invention are contemplated for use in pharmaceutical compositions. In one embodiment, a long acting EPO produced from oxidation of vicinal hydroxyls is included in the pharmaceutical composition of the invention. In another embodiment, the pharmaceutical composition of the invention includes at least one long acting EPO that is a result of replacing the sialic acid residues with less labile residues. In yet another embodiment, the long acting EPO included in the pharmaceutical composition is a result of increasing the negative charge on EPO by sulfation and/or phosphorylation. In still another embodiment, a long acting EPO produced by terminating the carbohydrate chains with more complex molecules, *e.g.*, PEG chains, is included in the pharmaceutical compositions of the invention.

In addition, the present invention contemplates the use of a mixture of long acting EPOs produced by any of the methods of the present invention in the pharmaceutical compositions of the invention. For example, the pharmaceutical composition of the invention may include at least one long acting EPO that is a result of replacing the sialic acid residues with less labile residues and at least one long acting EPO that is the result of increasing the negative charge on EPO by sulfation and/or phosphorylation.

Transport System

As discussed earlier, the long acting EPOs of the present invention advantageously are able to traverse barriers with capillaries having EPO receptors. Thus, another aspect of the present invention is a transport system using the long acting EPOs of the present invention as carriers for molecules with poor barrier penetration into a targeted area of the body having EPO receptors. Such transport systems advantageously provide a novel and safe method of delivery across the intact barriers.

In one embodiment, the transport system includes the long acting EPOs of the present invention and at least one molecule with poor brain penetration to provide a novel and safe method of delivery across the intact blood brain barrier. In other words, the long acting EPOs of the present invention may allow molecules with poor brain penetration to act as molecular "trojan horses" so as to enhance brain uptake of either small or large molecule diagnostics or therapeutic molecules.

In fact, an important problem in the treatment of human brain tumors is posed by the need to deliver therapeutic agents to specific regions of the brain, distributing them within and targeting them to brain tumors. The molecules that might otherwise be effective in diagnosis and therapy either do not cross the blood-brain barrier (BBB) in the brain adjacent to the tumor or do not cross the blood-

and primordial follicle cells; pancreas cells: islets of Langerhans, α-cells, β-cells, γ-cells, and F-cells; bone cells: osteoprogenitors, osteoclasts, and osteoblasts; skin cells; endometrial cells: endometrial stroma and endometrial cells; as well as the stem and endothelial cells present in the above listed organs.

Composition Blend of EPO Analog and Tissue Protective Cytokine

As briefly mentioned above, a pharmaceutical composition according to the present invention may include an EPO analog having at least one additional N-linked carbohydrate chain and/or at least one additional O-linked carbohydrate chain (that exhibits an extended serum half-life but lacks tissue protective activity) in a blend with at least one tissue protective cytokine. For example, an EPO analog having at least two additional N-linked carbohydrate chains, wherein one of the additional carbohydrate chains is located in the O'Brien peptide, in combination with a tissue protective cytokine, may form a composition of the invention. In another embodiment, the pharmaceutical composition of the invention may include at least one tissue protective cytokine and at least one EPO analog that contains additional carbohydrate chains that are known, from reviewing the analog in three-dimensional space, to block the O'Brien peptide. In yet another embodiment, a pharmaceutical composition of the invention includes at least one tissue protective cytokine and at least one EPO analog having no tissue protective functionality as a result of the method of adding the extra carbohydrate chains to the protein.

An EPO analog with a relocated glycoylation site is contemplated for use in the pharmaceutical compositions of the present invention. Without being bound to any particular theory, it is believed that if the naturally occurring glycosylation site at amino acid 38 was relocated elsewhere on an EPO analog, outside of the 30-47 amino acid segment, the tissue protective capabilities of the EPO analog would be enhanced as compared to an EPO analog with the glycosylation site at amino acid 38. Thus, the pharmaceutical composition of the invention may include an EPO analog with a relocated glycosylation site, from the 38 amino acid, to elsewhere on the molecule. The relocated glycosylation site may occur at amino acids 51, 57, 69, 88, 89, 136 or 138, as suggested in PCT Publication No. WO 01/81405. In one embodiment, the O'Brien peptide contains 1 or less carbohydrate chains. In an alternative embodiment, the O'Brien peptide includes 2 or more carbohydrate chains.

Suitable tissue protective cytokines for use with this aspect of the present invention are preferably those cytokines that lack an effect on the bone marrow but maintain the tissue protective

K140A/K52A/K45A, K97A/K152A, K97A/K152A/K45A, K97A/K152A/K45A/K52A, K97A/K152A/K45A/K52A/K140A, K97A/K152A/K45A/K52A/K140A/K154A, N24K/N38K/N83K, and N24K/Y15A. In yet another embodiment, the tissue protective cytokines do not include any of the above combinations. In another embodiment, the tissue protective cytokines may include any of the above-referenced site mutations providing that the site mutations do not include any of the following combinations of substitutions: N24K/N38K/N83K and/or A30N/H32T.

Certain modifications or combinations of modifications may affect the flexibility of the mutein's ability to bind with its receptor, such as an EPO receptor or secondary receptor. Examples of such modifications or combinations of modifications include, but are not limited to, K152W, R14A/Y15A, I6A, C7A, D43A, P42A, F48A, Y49A, T132A, I133A, T134A, N147A, P148A, R150A, G151A, G158A, C161A, and R162A. Corresponding mutations are known to those of ordinary skill in the art to be detrimental in human growth hormone. Thus, in one embodiment, the tissue protective cytokine does not include one or more of the modifications or combinations of modifications that may affect the flexibility of the mutein's ability to bind with its receptor. Further discussion of such tissue protective cytokines is included in co-pending U.S. Patent Application No. ____, attorney docket no. 10165-022-999, filed July 1, 2003, entitled "Recombinant Tissue Protective Cytokines and Encoding Nucleic Acids Thereof for Protection, Restoration, and Enhancement of Responsive Cells, Tissues, and Organs," the entire disclosure of which is incorporated by reference herein

Finally, any of the superfamily cytokines that exhibit tissue protective capabilities may be used as well so long as they do not interfere with the long acting EPO's erythropoietic effects or serum half-life. Examples include, but are not limited to, interleukin-3 (IL-3), interleukin-5 (IL-5), granulocyte-macrophage colony-stimulating factor (GMCSF), pigment-epithelium derived factor (PEDF), and vascular endothelial growth factor (VEGF).

In another aspect of the present invention, a pharmaceutical composition according to the present invention may include an EPO analog having at least one additional N-linked carbohydrate chain and/or at least one additional O-linked carbohydrate chain (that exhibits an extended serum half-life but lacks tissue protective activity) in a blend with at least one small molecule that exhibits tissue protective functionality. Suitable small molecules include, but are not limited to, steroids (*e.g.*, lazaroids and glucocorticoids), antioxidants (*e.g.*, coenzyme Q_{10}, alpha lipoic acid, and NADH), anticatabolic enzymes (*e.g.*, glutathione peroxidase,superoxide dimutase, catalase, synthetic catalytic scavengers, as well as mimetics), indole derivatives (*e.g.*, indoleamines, carbazoles, and carbolines),

administration, acute treatment, and/or intermittent administration. In one embodiment, the pharmaceutical compositions of the invention are administered chronically to protect or enhance the target cells, tissue or organ. In another embodiment, the pharmaceutical compositions of the invention may be administered acutely, *i.e.*, for a single treatment during injury. In yet another embodiment, the pharmaceutical compositions of the invention are administered in a cyclic nature.

The administration of the composition may be parenteral, *e.g.*, via intravenous injection, intraperitoneal injection, intra-arterial, intramuscular, intradermal, or subcutaneous administration; via inhalation; transmucosal, *e.g.*, oral, nasal, rectal, intravaginal, sublingual, submucosal, and transdermal; or combinations thereof. Preferably, the administration of the pharmaceutical composition of the invention is parenteral. Such adminstration may be performed in a dose amount of about 0.01 pg to about 5 mg, preferably about 1 pg to about 5 mg. In one embodiment, the dose amount is about 500 pg to about 5 mg. In another embodiment, the dose amount is about 1 ng to about 5 mg. In yet another embodiment, the dose amount is about 500 ng to about 5 mg. In still another embodiment, the dose amount is about 1 μg to about 5 mg. For example, the dose amount may be about 500 μg to about 5 mg. In another embodiment, the dose amount may be about 1 mg to about 5 mg.

Pharmaceutical compositions of the invention adapted for parenteral administration include aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain antioxidants, buffers, bacteriostats and solutes that render the compositions substantially isotonic with the blood of an intended recipient. In this aspect of the invention, the pharmaceutical compositions may also include water, alcohols, polyols, glycerine, vegetable oils, and mixtures thereof. Pharmaceutical compositions adapted for parenteral administration may be presented in unit-dose or multi-dose containers, for example sealed ampules and vials, and may be stored in a lyophilized (freeze-dried) condition requiring only the addition of a sterile liquid carrier, *e.g.*, sterile saline solution for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets. In one embodiment, an autoinjector comprising an injectable solution of a long acting EPO of the invention may be provided for emergency use by ambulances, emergency rooms, and battlefield situations.

In one embodiment, the pharmaceutical composition of the invention is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. For example, the pharmaceutical composition may be in the form of a solution in sterile isotonic aqueous buffer. Where necessary, the pharmaceutical composition may

thereof. When the topical administration is intended for the skin, mouth, eye, or other external tissues, a topical ointment or cream is preferably used. And, when formulated in an ointment, the active ingredient, *i.e.*, the long acting EPO, may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water base or a water-in-oil base. When the topical administration is in the form of eye drops, the pharmaceutical compositions of the invention preferably include the active ingredient, which is dissolved or suspended in a suitable carrier, *e.g.*, in an aqueous solvent.

Pharmaceutical compositions adapted for nasal and pulmonary administration may include solid carriers such as powders (preferably having a particle size of about 20 microns to about 500 microns). Powders may be administered by rapid inhalation through the nose from a container of powder held close to the nose. In an alternate embodiment, pharmaceutical compositions intended for nasal administration according to the present invention may include liquid carriers, *e.g.*, nasal sprays or nasal drops. Preferably, the pharmaceutical compositions of the invention are administered into the naval cavity directly.

Direct lung inhalation may be accomplished by deep inhalation through a mouthpiece into the oropharynx and other specially adapted devices including, but not limited to, pressurized aerosols, nebulizers or insufflators, which can be constructed so as to provide predetermined dosages of the active ingredient. Pharmaceutical compositions intended for lung inhalation may include aqueous or oil solutions of the active ingredient. Preferably, the pharmaceutical compositions of the invention are administered via deep inhalation directly into the oropharynx.

Pharmaceutical compositions adapted for rectal administration may be provided as suppositories or enemas. In one embodiment, the suppositories of the invention includes about 0.5 percent to 10 percent by weight of active ingredient. In another embodiment, the suppository includes about 1 percent to about 8 percent by weight active ingredient. In still another embodiment, the active ingredient is present in the suppository in an amount of about 2 percent to about 6 percent by weight. In this aspect of the invention, the pharmaceutical compositions of the invention may include traditional binders and carrier, such as triglycerides.

Pharmaceutical compositions adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

The pharmaceutical compositions of the invention may also be administered by use of a perfusate, injection into an organ, or locally administered. In such embodiments, the pharmaceutical composition preferably has about 0.01 pM to about 30 pM, preferably about 15 pM to about 30 nM,

Dosing

Selection of the preferred effective and non-toxic dose for the administration methods above will be determined by a skilled artisan based upon factors known to one of ordinary skill in the art. Examples of these factors include the particular form of long acting EPO; the pharmacokinetic parameters of the EPO, such as bioavailability, metabolism, half-life, etc. (provided to the skilled artisan); the condition or disease to be treated; the benefit to be achieved in a normal individual; the body mass of the patient; the method of administration; the frequency of administration, *i.e.*, chronic, acute, intermittent; concomitant medications; and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus the precise dosage should be decided according to the judgment of the practitioner and the circumstances of the particular patient.

For example, the Physicians Desk Reference (PDR) shows that, depending on the patient population being treated with EPO, different hematocrit levels are targeted to avoid toxicity. Physicans Desk Reference, 54th Ed., 519-525 and 2125-2131 (2000). In fact, in patients with CRF, the PDR recommends dosing EPO to achieve non-toxic target hematocrits ranging from 30 percent to 36 percent. In contrast, for cancer patients on chemotherapy, the PDR teaches to adjust the dosage at a different hematocrit level, *i.e.*, if the hematocrit level exceeds 40 percent. The PDR shows that practitioners monitor the patient's hematocrit during therapy with EPO and, to avoid toxicity, adjust the dose and/or withhold treatment if the patient's hematocrit approaches or exceeds the upper limits of a target range. Therefore, the skilled practitioner, armed with the teachings of the present invention, should be able to administer doses of EPO sufficient to achieve a therapeutic effect while avoiding any toxicity complications.

In one embodiment, the long acting EPO of the present invention is administered chronically or systemically at a dosage of about 0.1 μg/ kg body weight to about 100 μg /kg body weight per administration. For example, about 1 μg/ kg body weight to about 5 μg/ kg body weight is contemplated for once weekly dosing in the treatment of cancer patients receiving chemotherapy. In another embodiment, the dosage of the long acting EPO is about 5 μg /kg body weight to about 50 μg /kg-body weight per administration. In still another embodiment, the long acting EPO is administered in an amount of about 10 μg /kg body weight to about 30 μg /kg body weight per administration. In yet another embodiment, the long acting EPO is administered in an amount of about 1 μg/ kg body weight or less. For example, about 0.45 μg/ kg body weight to about 0.75 μg/ kg body weight of long acting EPO may be effective when administered once weekly for treatment of anemia in CRF patients.

specific amount of carrier for each treatment session. In another embodiment the kit may contain a plurality of ampules each containing specific amounts of the lyophilized material and a plurality of containers each containing specific amounts of carrier, such that the administrator need only mix the contents of one ampule and one carrier container for each treatment session without measuring or weighing. In yet another embodiment, the kit contains an autoinjector including an injectable solution of a long acting EPO of the invention. In still another embodiment, the kit contains at least one ampule with the lyophilized composition, at least one container of carrier solution, at least one container with a local anesthetic, and at least one syringe (or the like). The ampules and containers are preferably hermetically-sealed.

When the pharmaceutical compositions of the invention are to be administered by infusion, the kit preferably includes at least one ampule with the pharmaceutical composition and at least one infusion bottle with sterile pharmaceutical grade water or saline.

A kit according to the present invention may also include at least one mouthpiece or specially adapted devices for direct lung inhalation such as pressurized aerosols, nebulizers, or insufflators. In this aspect of the invention, the kit may include the device for direct lung inhalation, which contains the pharmaceutical composition, or the device and at least one ampule of aqueous or oil solutions of the long acting EPO of the present invention.

When the long acting EPO pharmaceutical composition of the invention is adapted for oral, transdermal, rectal, vaginal, or nasal, the kit preferably includes at least one ampule containing the active ingredient and at least one administration aid. Examples of administration aids include, but are not limited to, measuring spoons (for oral administration), sterile cleaning pads (for transdermal adminstration, and nasal aspirators (for nasal administration). Such kits may include a single dose of the long acting EPO (acute treatment) or a plurality of doses (chronic treatment).

In addition, the kit may be outfitted with one or more types of solutions. For example, the long acting EPO pharmaceutical compositions of the invention may be made in an albumin solution and a polysorbate solution. If the kit includes the polysorbate solution, the words "Albumin free" preferably appear on the container labels as wells as the kit main panels.

Moreover, the kit may also include a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

determining erythropoietic activity are available. For example, European Pharmocopeia discusses at least two assays useful in determining the erythropoietic activity of an EPO compound, which include exhypoxic mouse assays and reticulocyte assays.

Tissue Protective Capability Assays Based on EPO Receptor

In one embodiment, the tissue protective capability assays of the present invention are based on the tissue protective receptor for EPO. Once the sequence for the tissue protective receptor is isolated, a variety of assays may be used to determined a particular EPO compound's tissue protective capability. As known to those of ordinary skill in the art, the type of assay employed largely depends on the weight of the EPO compound.

For example, the assays may be competitive assays or sandwich assays or steric inhibition assays. Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. As used herein, the term "analyte" refers to the EPO compound of interest to be tested for tissue protective activity. The term "binding partner" refers to any protein that binds to the analyte (typically the EPO receptors). As used herein, "tracer" refers to labeled reagants, such as labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner and steric conjugates. The tracer used herein may be any detectable functionality that does not interfere with the binding of analyte and its binding partner. Nonlimiting examples include moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties that must be reacted or derivatized to be detected, such as enzymes. Suitable tracers may be the radioisotopes P^{32}, C^{14}, I^{125}, H^3, I^{131}, and mixtures thereof; fluorophores, such as rare earth chelates, fluorescein, fluorescein derivatives, rhodamine, rhodamine derivatives, dansyl, umbelliferone luciferase (firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456)), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (uricase and xanthine oxidase) coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, microperoxidase, and mixtures thereof; biotin/avidin; spin labels; bacteriophage labels; stable free radicals; and combinations thereof. In one embodiment, the tracer is at least one of horseradish peroxidase or alkaline phosphatase.

A skilled artisan is aware of methods of covalently binding the tracers to proteins or

conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte such that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. The analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, *e.g.*, a change in fluorescence when the hapten is a fluorophore.

More information regarding tissue protective capability assays is discussed in co-pending U.S. Patent Application No. 10/188,905, filed July 3, 2002 and in Application Serial No. 60/456,891, filed April 25, 2003, both of which are incorporated by reference herein in their entireties.

Functional Assays

In the absence of the identification of the sequence for the tissue protective receptor, the tissue protective capabilities of an EPO may be determined using functional assays, both *in vivo* and *in vitro*. Preferably, one of ordinary skill in the art would perform a single *in vitro* or *in vivo* assay to determine the tissue protective capabilities of an EPO compound, but in certain instances it may be necessary to perform both to assure that the compound exhibits the same tissue protective capabilities *in vitro* and *in vivo*.

In practice, one of ordinary skill in the art would be able to determine whether an EPO analog having at least one additional N-linked carbohydrate chain and/or at least one additional O-linked carbohydrate chain, using a combination of assays disclosed by the present invention. First, *in vitro* tests such as the P19 cell and rat motoneuron assays could be used to determine whether the EPO compound of interest exhibited tissue protective capabilities. Then, *in vivo* studies such as the rat focal ischemia, bicuculline seizure, or spinal cord trauma models could be used to verify the results of the *in vitro* testing.

In vitro models contemplated by the present invention include, but are not limited, to those used to determine the lack of tissue protective capabilities of the hyperglycosylated-erythropoietin above: the P19 cell assay, rat motoneuronal cell assay, and the cDNA microarray, which are discussed in greater detail below and further illustrated in Example 2. The examples are intended to be non-limiting as one of ordinary skill in the art would recognize that there are other suitable *in vitro* assays for determining the tissue protective capabilities of EPO compounds. In general, the EPO compound would be considered tissue protective if, in comparison to a control, it maintained or enhanced the viability of the cell. The erythropoietin would be considered antagonistic if, in comparison to the control, it detrimentally affected the viability of the cells within the assay.

blocked with 10 percent fetal calf serum in PBS, incubated with antibodies against non-phosphorylated neurofilaments (SMI-32; 1:9000) overnight, and visualized using the avidin-biotin method with diaminobenzidine. The viability of motoneurons may be assessed morphologically by counting SMI-32 positive cells.

Mixed primary cultures of motoneurons characteristically undergo apoptosis during maintenance culture conditions. Addition of recombinant EPO (10 U/ml) to the culture medium 5 days before assessment of cell number has been shown to significantly increase the number of primary motoneurons observed at 5 days. Thus, to be considered tissue protective, the EPO compound of interest preferably salvages at least the same number of motoneurons than the control. In one embodiment, the EPO compound of interest is considered tissue protective if a greater number of motoneurons are saved from death during maintenance culture conditions as compared to the control.

C. *In Vitro* Assay Based on cDNA Microarray

Another *in vitro* assay to determine tissue protective capability of an EPO compound of interest is a cDNA microarray. This assay may be used to determined if recombinant EPO and the EPO compound of interest modify gene expression differently in P19 cells. mRNA isolated from undifferentiated P19 cells can show a different pattern of gene modulation estimated from a mouse 1200 cDNA microarray, depending upon the exposure to the EPOs. For example, the expression of 1,200 genes in P19 cells may be measured by the use of nylon membrane arrays from Clontech (Atlas mouse 1.2). Cells (10^7/sample) may be treated overnight with saline, recombinant EPO, an EPO compound of interest (1mU/ml), or mixtures thereof. The cells are then lysed for RNA extraction or subjected to serum deprivation for 3 hours (always in the presence of the same cytokine added during pretreatment). After standard total RNA extraction by column chromatography, with on-column DNase treatment, polyA + RNA may be purified. Probes may then be constructed in the presence of [P^{32}]-ATP. The labeled probes, having preferably 20 million counts or higher, may be hybridized to the cDNA nylon membranes at 68° C. The membranes are washed and exposed to x-ray film. The intensity of radioactive signals may be measured with a Phosphor Imager and analyzed with the Atlas Image 2.0 computer program (Clontech).

In vivo assays contemplated by the present invention include, but are not limited to, the tissue protective assays used to evaluate EPO compounds such as the focal ischemia model and intra-hippocampal biculline model. In addition, an *in vivo* model for evaluating tissue protection includes

electrodes (60 μm) may then be implanted bilaterally into the denate gyrus of the dorsal hippocampus (septal pole) and a cannula (22-gauge) may be unilaterally positioned on top of the dura for the intrahippocampal or intracerebroventricular infusion of drugs. The coordinates from bregma for implantation of the electrodes should be: (mm) antero-posterior-3.5; lateral 2.4 and 3 below dura with the nose bar set at -2.5. Paxinos, G. & Watson, C., *The Rat Brain in Stereotaxic Coordinates*, Academic Press, New York (1986). The electrodes may be connected to a multipin socket (March Electronics, NY) and, together with the injection cannula, secured to the skull by acrylic dental cement.

The experiments are preferably carried out three to seven days after surgery when the animals have fully recovered. Animals are then administered recombinant EPO or the EPO compound of interest (both 5000 U/kg-bw) or vehicle intraperitoneally 24 hours and again at 30 minutes before the induction of bicuculline seizures. The procedures for recording the EEG and intracerebral injection of drugs have been previously described Vezzani, A., *et al.*, *J. Pharmacol Exp Ther*, **239**, 256-63 (1986). Briefly, the animals are allowed to acclimatize in a Plexiglass cage (25x25x60 cm) for a minimum of 10 minutes before initiating the EEG recording (4-channel EEG polygraph, model BP8, Battaglia Rangoni, Bologna, Italy). After about 15 minutes to about 30 minutes, EEG recordings are made continuously for 120 minutes after 0.8 nmol/0.5 μl bicuculline methiodide infusion. All the injections were made to unanesthetised rats using a needle (28-gauge) protruding 3 mm below the cannula.

Seizures may be measured by EEG analysis, which has previously been shown to provide a sensitive measure of the anticonvulsant activity of drugs. Vezzani, A., et al., *J. Pharmacol Exp Ther*, **239**, 256-63 (1986). For the purposes of this assay, seizures consist of the simultaneous occurrence of at least two of the following alterations in all four leads of recordings: high frequency and/or multispike complexes and/or high voltage synchronized spike or wave activity. Synchronous spiking may be observed intermixed with seizures. The parameters chosen to quantify seizures are preferably the latency to the first seizure (seizure onset), the total time spent in epileptic activity (determined by adding together the duration of ictal episodes; seizure duration), and the spiking activity during the EEG recording period (seizure activity).

The intra-hippocampal bicuculine seizure model using EEG activity as a read-out has been shown to be a sensitive and specific predictor of anti-seizure potency of drugs. Vezzani, A., et al., *J. Pharmacol Exp Ther*, **239**, 256-63 (1986). Thus, to be considered tissue protective the EPO compound of interest should reduce the frequency and severity of the seizures to the same or greater

H. Spinal Cord Injury Assays

Spinal cord injury assays may also be used with the present invention to evaluate the tissue protective abilities of particular EPO compounds of interest. In particular, rat spinal cord compression is contemplated for use with the present invention. Wistar rats (female) weighing about 180 g to about 300 g are preferably used in this study. The animals are preferably fasted for 12 hours before surgery, and humanely restrained and anesthesized with an intraperitoneal injection of thiopental sodium (40 mg/kg-bw). After infiltration of the skin (bupivacaine 0.25 percent), a complete single level (T-3) laminectomy is performed through a 2 cm incision with the aid of a dissecting microscope. Traumatic spinal cord injury is induced by the extradural application of a temporary aneurysm clip exerting a 0.6 newton (65 grams) closing force on the spinal cord for 1 minute. After removal of the clip, the skin incision is closed and the animals allowed to recover fully from anethesia and returned to their cages. The rats are monitored continuously with bladder palpation at least twice daily until spontaneous voiding resumed.

Animals in a control group receive normal saline (via intravenous injection) immediately after the incision is closed. The remaining animals receive the EPO compound of interest in an amount of 16 micrograms/kg-bw iv. The motor neurological function of the rats is then evaluated using a locomotor rating scale. In this scale, animals are assigned a score ranging from 0 (no observable hindlimb movements) to 21 (normal gait). The rats are tested for functional deficits at 1 hour, 12 hours, 24 hours, 48 hours, 72 hours, and 1 week after injury by the same examiner who is blind to the treatment each animal receives. If the EPO compound of interest is tissue protective, the rats that are given the EPO should exhibit a quicker and beter overall recovery from the injury than the rats that are given the saline injection.

I. Rabbit Spinal Cord Ischemia Testing

In another embodiment, rabbit spinal cord ischemia testing allows testing for tissue protective capability. For example, New Zealand White rabbits (36, 8-12 months old, male) weighing 1.5 kg to 2.5 kg are used in this study. The animals are fasted for 12 hours and humanely restrained. Anesthesia induction is via 3 percent halothane in 100 percent oxygen and maintained with 0.5 percent to 1.5 percent halothane in a mixture of 50 percent oxygen and 50 percent air. An intravenous catheter (22 gauge) is placed in the left ear vein. Ringers lactate is infused at a rate of 4 ml/kg body weight (bw) per hour during the surgical procedure. Preoperatively, cefazoline 10 mg/kg-bw is administered intravenously for prophylaxis of infection. The animals are placed in the

described above. In addition, to evaluate an EPO analog's effect upon myocardial cells, a skilled artisan could readily modify the myocardial infarction model discussed above. Those of ordinary skill in the art will be sufficiently skilled to select the appropriate assay or model to evaluate whether a particular EPO possesses tissue protective activities with regard to an erythropoietic responsive cell, tissue or organ.

EXAMPLES

The following prophetic examples are merely illustrative of the preferred embodiments of the present invention, and are not to be construed as limiting the invention, the scope of which is defined by the appended claims.

Example 1: Chemically Modified EPO

A. Oxidation of Sugar Chains

The sugar units of EPO may be converted into acids by the following procedure. EPO and an amount of sodium periodate sufficient to provide the amount of oxidation desired (the greater the amount of sodium periodate the greater the extent of the oxidation) may be placed within a 100 mM sodium acetate buffer. This solution may then be incubated on ice for about 20 minutes and dialyzed thoroughly using distilled water. The product may then be removed from the dialysis tubing and collected into a fresh tube (Product I).

A Quantitative Benedict Solution (18 g copper sulfate, 100g sodium carbonate (anhydrous), 200 g potassium citrate, 125 g potassium thiocyanante, 25 g potassium ferrocyanide) may be dissolved into distilled water to a final volume of 1 liter. Several drops of methylene blue may then be added to the Quantitative Benedict Solution.

Product I may then be added to the Quantitative Benedict Solution until the color of the solution becomes clear indicating the solution is fully oxidized. The solution may then be desalted and concentrated using an Ultrafree Centrifugal Filter Unit. The sample (Product II) may then be further dialyzed thoroughly using distilled water.

B. Oxidation of Asialo Form EPO with Galactose Oxidase

50 to 500 μg asialo form of EPO , 10 μl 1U/μl galactose oxidase, and 100 μl 10 mM sodium phosphate buffer may be mixed in a 15 ml conical centrifuge tube (110 μl total volume). This mixture may then be incubated for 2 hours at 37°C, at which time the solution may be dialyzed

E. Attachment of PEG chains to Asialo EPO

An asialo form of EPO may be modified through the attachment of PEG chains to newly created terminal galactose residues after oxidiation with galactose oxidase, such as those obtained above in B (Product III).

Recombinant human EPO (rhuEPO) may be desialized using Sialidase A (Prozyme, Inc.) according to the manufacturer protcol. The chemical modification is preferably confirmed by running the reaction product on a SDS polyacrylamide gel. Staining the resultant bands should show that the modified EPO has an apparent molecular weight of about 31 kDa, while the unmodified EPO has a molecular weight of about 34 kDa. The sialic acid residues remaining on the EPO are preferably less than 0.1 mole/mole of EPO.

After the asialo form of EPO is obtained, the newly exposed galactose residues on EPO (2-4 mg/ml in 10 mM sodium phosphate buffer) may be oxidized with 100 units of galactose oxidase in PBS (Sigma) per ml of EPO solution. The reaction mixture may then be incubated at 37°C for 2 hours.

The phosphate buffer may then be removed by buffer exchange in 100 mM sodium acetate, ph 5.4. Methoxy-PEG-hydrazide of various molecular weights (Nektar Therapeutics) may then be added at a 5 fold to 100 fold molar excess (polymer: protein). The intermediate hydrazine linkage is then preferably further reduced by the addition of 15 mM sodium cyanoborohydride (Sigma) and allowed to react overnight at 4°C. The resultant conjugates may then be fractionated / purified by techniques known in the art.

F. Attachment of PEG chains to Asialo EPO

An asialo form of EPO may be modified through the attachment of PEG chains to newly created terminal galactose residues after oxidiation with galactose oxidase, such as those obtained above in B (Product III).

RhuEPO (1 mg) may be desialized using Neuraminidase (Seikagaku Corporation of Japan, 1 U of lyophilized powder is dissolved in 100 μL of 75 mM $NaPO_4$ (pH 6.5)) at a ratio of 1mg EPO to 0.05 units of Neuraminidase (5 μL). Five units (5 μL) of galactose oxidase (450 μL dissolved in 75 mM $NaPO_4$ (pH 6.5) (Sigma)) may then be added to the mixture.

The phosphate buffer may then be removed by buffer exchange in 100 mM sodium acetate, ph 5.4. PEG-NH_2 (750 molecular weight, Nektar Therapeutics) and 15 mM sodium

The tissue protective attributes of an EPO analog having at least one additional N-linked carbohydrate chain and/or at least one additional O-linked carbohydrate chain were determined using the following assay.

Neuronal cultures were established from the hippocampus of 18-day rat fetuses. Brains were removed and freed from meninges and the hippocampus was isolated. Cells were then dispersed by incubation for 5 minutes at 37° C in a 2.5 percent trypsin solution followed by titration. The cell suspension was diluted in serum-free Neurobasal media containing 1 percent B-27 supplement (Gibco, Rockville, MD, USA) and plated onto polyornithine-coated coverslips at a density of 80,000 cells per coverslip. Cells were then pre-treated with EPO overnight and then exposed with or without 1) EPO, 2) an EPO analog having at least one additional N-linked carbohydrate chain and/or at least one additional O-linked carbohydrate chain, or 3) an asialo form of EPO to 5 μM TMT for 24 hours. Cultures were used between 10 and 14 days *in vitro*.

The viability of the cells was measured by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Denziot, F., and Lang, R. 1986. Rapid Colormetric Assay for Cell Growth and Survival. Modifications to the tetrazolium dye procedure giving improved reliability. *J Immunol Methods* **89**: 271-277. Briefly, MTT tetrazolium salt was dissolved in serum-free medium to a final concentration of 0.75 mg/ml and added to the cells at the end of the treatment for 3 hours at 37° C. The medium was then removed and the formazan was extracted with 1N HCl:isopropanol (1:24) . Absorbance at 560 nm was read on a microplate reader.

As demonstrated in FIG. 1A, the EPO analog having at least one additional N-linked carbohydrate chain and/or at least one additional O-linked carbohydrate chain did not exhibit a tissue protective function.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. All references cited herein are incorporated by reference herein in their entireties for all purposes.

protective functionality.

8. The erythropoietin product of claim 6, wherein the at least one chemical modification comprises oxidation of at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain to provide at least one additional acid residue.

9. The erythropoietin product of claim 6, wherein the at least one chemical modification comprises sulfation of at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain to provide an increased negative charge on the EPO product.

10. The erythropoietin product of claim 6, wherein the at least one chemical modification comprises phosphorylation of at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain to provide an increased negative charge on the EPO product.

11. The erythropoietin product of claim 6, wherein the at least one chemical modification comprises addition of at least one polyethylene glycol chain to at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain.

12. A method for preparing an erthyropoietin product having an extended serum half-life and tissue protective activity comprising the steps of:

providing at least one erythropoietin or erythropoietin derivative; and

modifying at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain on the at least one endogenous or recombinant erythropoietin by oxidation, sulfation, phosphorylation, PEGylation, or a combination thereof.

13. The method of claim 12, wherein the step of modifying further comprises the step of replacing at least one vicinal hydroxyl on at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain with at least one acid residue.

14. The method of claim 13, wherein the step of replacing at least one vicinal hydroxyl on at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain with at least one acid residue further comprises replacing a plurality of vicinal hydroxyls on the least one N-linked

19. A method for treating anemia in patients at risk for tissue damage comprising the steps of:
providing an erythropoietin product with at least one chemical modification to at least one of the N-linked oligosaccharide chains or the O-linked oligosaccharide chain, wherein the chemical modification comprises oxidation, sulfation, phosphorylation, PEGylation, or a combination thereof;
administering a therapeutically effective amount of the erythropoietin product, wherein the erythropoietin product is administered at a lower molar amount than rhuEPO to obtain a comparable target hematocrit,
wherein the erythropoietin product has tissue protective functionality.

20. The method of claim 19, wherein the erythropoietin product has a longer serum half-life than rhuEPO.

21. The method of claim 20, wherein the serum half-life is at least about 20 percent longer than the serum half-life of rhuEPO.

22. The method of claim 21, wherein the serum half-life is at least about 40 percent longer than the serum half-life of rhuEPO.

23. A pharmaceutical composition comprising:
a therapeutically effective amount of at least one erythropoietin derivative, wherein at least one N-linked oligosaccharide chain or at least one O-linked oligosaccharide chain has at least one chemical modification as a result of oxidation, sulfation, phosphorylation, PEGylation, or mixtures thereof,
wherein the at least one erythropoietin derivative has a longer serum half-life than recombinant erythropoietin and has tissue protective functionality.

24. The pharmaceutical composition of claim 23, further comprising at least one pharmaceutically acceptable carrier.

25. The pharmaceutical composition of claim 24, wherein the at least one pharmaceutically

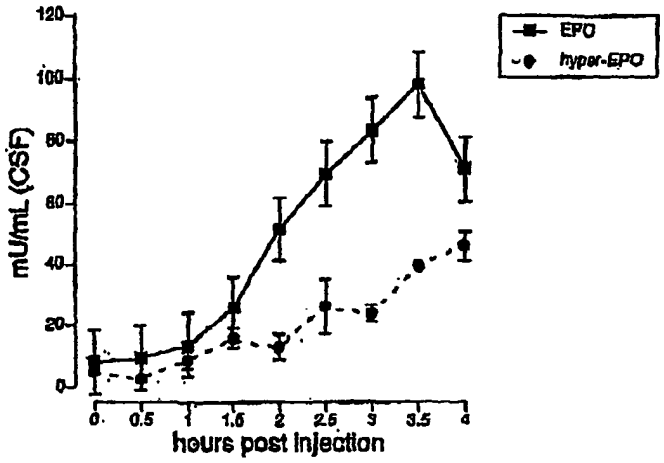

Figure 1A

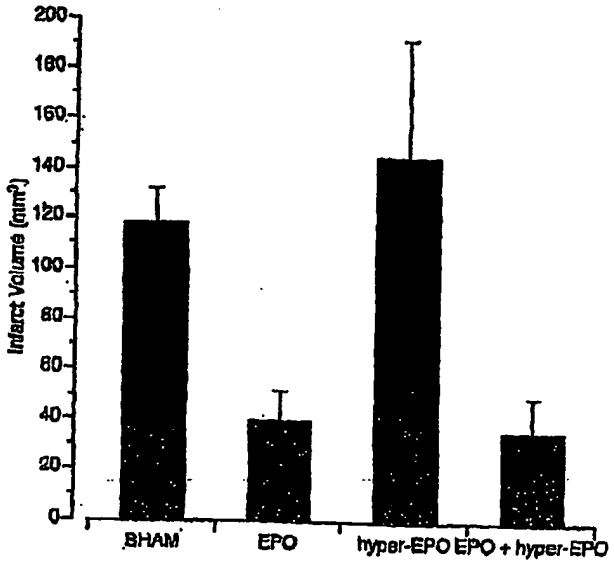

Figure 1B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/28073

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 38/18, 38/16, 38/22; C07K 1/10, 14/505
US CL : 514/8, 12, 814; 530/397, 402, 408, 409, 410, 411
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/8, 12, 814; 530/397, 402, 408, 409, 410, 411

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	US 6,340,742 B1 (BURG et al.) 22 January 2002 (22.01.02), abstract, claim 1, Col. 15, Example 2, Fig. 3.	1-2, 4-9, 11-12, 15, 17-26 --- 1-27
X --- A	EGRIE et al. Development and Characterization of novel erythropoiesis stimulating protein (NESP). Nephrol. Dial. Transplant. (2001) Vol. 16, Suppl. 3, pp. 3-13, see especially Fig. 1, Fig. 2-6, Fig. 8-9, pp. 9-12.	1, 3-5 --- 1-26
X	HANSEN et al. A Randomized, Blinded Placebo-controlled, Phase II, Dose-Finding Study of ARANESP in Patients with lymphoproliferative malignancies. Blood, November 2000, Vol. 96, No. 11, p. 155b.	1, 3-5
A	VANSTEENKISTE et al. Darbepoetin alfa: a new approach to the treatment of chemotherapy-induced anaemia. Expert. Opin. Biol. Ther. 2003, Vol. 3, No. 3, pp. 501-508.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
21 February 2004 (21.02.2004)

Date of mailing of the international search report
14 JUL 2004

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Facsimile No. (703) 305-3230

Authorized officer
Holly Schnizer
Valerie Bell-Harris for
Telephone No. 571/272-1600

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US03/28073

Continuation of B. FIELDS SEARCHED Item 3:
STN (Bioscience); EAST (all databases); search terms: erythropoietin, EPO, darbepoeitin, half-life, protective, erythropoiesis, oxidized, PEG, pegylated, glycosylat?, phosphorylat?, sulfonat?.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 20528.0006.W	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US03/28073	International filing date *(day/month/year)* 09 September 2003 (09.09.2003)	Priority date *(day/month/year)* 09 September 2002 (09.09 2002)
International Patent Classification (IPC) or national classification and IPC IPC(7): A61K 38/18, 38/16, 38/22; C07K 1/10, 14/505 and US Cl.. 514/8, 12, 814; 530/397, 402, 408, 409, 410, 411		
Applicant WARREN PHARMACEUTICALS, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of ___ sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of report with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02 April 2004 (02.04.2004)	29 March 2005 (29.03.2005)
Name and mailing address of the IPEA/US Mail Stop PCT, Attn: IPEA/ US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Holly Schnizer Telephone No. (703 308-0196

Form PCT/IPEA/409 (cover sheet)(July 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US03/28073

I. Basis of the report

1. With regard to the elements of the international application:*

☒ the international application as originally filed.

☒ the description:
pages 1-49 as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the claims:
pages 50-54, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the drawings:
pages 1-1, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☐ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of

☐ the description, pages NONE

☐ the claims, Nos. NONE

☐ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US03/28073

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-27	YES
	Claims NONE	NO
Inventive Step (IS)	Claims 1-27	YES
	Claims NONE	NO
Industrial Applicability (IA)	Claims 1-27	YES
	Claims NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-27 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a man-made erythropoietin product comprising the claimed modifications to the oligosaccharide chains wherein the erythropoietin has a longer serum half-life and tissue protective functionality or methods of using such erythropoietin products. The cited references teach modified EPO proteins with additional glycosylation sites that have increased half-lives. The cited references do not discuss any tissue protective activity of the proteins disclosed therein. In light of the evidence in the description indicating that modified EPO proteins with additional glycosylation sites did not have tissue protective activity, it appears that tissue protective activity would not necessarily be a property of the EPO proteins disclosed in the cited references.

Claims 1-27 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US03/28073

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claims 1-27 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because the claims are not fully supported by the description. The application, as originally filed, did not describe:the entire genus of erythropoietin molecules that have longer serum half-lives than rhuEPO and tissue protective functionality. The application also does not describe the entire genus of erythropoietin molecules with the claimed modified oligosaccharide chains that would have the claimed activity. A description of a genus may be achieved by means of a recitation of a representative number of species falling within the scope of the genus or a recitation of structural features common to members of the genus, which features constitute a substantial portion of the genus. A generic statement that defines a genus of proteins only by their functional activity does not provide adequate written description. In the case of the claims limited to modifications of oligosaccharide chains, these claims do not provide limitations as to which oligosaccharide chains (including oligosaccharide chains that could be added to the erythropoietin) could be modified to successfully achieve the claimed activities or any limitations on amino acid sequences and the specification does not provide any guidance as to any particular modified EPO that would meet the limitations of the claims. Thus, the genus is enormous and the description does not provide a single species of this genus. Thus, a representative number of species falling within the scope of the genus has not been described and the description is considered to lack adequate written description for the claimed genus.

Claims 1-27 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because the claims are not fully supported by the description. The description does not disclose the claimed invention in a manner sufficiently clear and complete for the claimed invention to be carried out by a person skilled in the art because the description does not teach how to make a man-made erythropoietin product with the claimed activities. The effect of structural changes on protein function especially in vivo is highly unpredictable. In fact, the present description indicates that it was surprising that an EPO analog with an extra glycosylation site at the 32 amino acid, which results in a longer half-live, does not have tissue protective activity (see p. 11 of the Description). The Specification does not provide any guidance as to what modifications can be made to EPO oligosaccharide chains that would successfully provide the claimed acitivities and has not even provided a single example of an EPO modified at the oligosaccharide chains that has the activities as claimed. Therefore, due to the unpredictability of making modified EPO proteins that would have the claimed activities, the lack of guidance or examples of modifications that would successfully result in the claimed activities, the lack of any teachings in the prior art as to how to make the molecules of the claimed genus, it would require undue experimentation to make the modified EPO proteins of the claims. If follows that if the description has not sufficiently taught how to make the products that the methods of using the products also lack enablement.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
JOHN P. MULGREW
SWIDLER BERLIN SHEREFF FRIEDMAN, LLP
3000 K STREET, N.W.
SUITE 300
WASHINGTON, DC 20007-5116

RECEIVED
APR 27 2005
SWIDLER BERLIN LLP

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

	Date of Mailing *(day/month/year)* 26 APR 2005
Applicant's or agent's file reference 20528.0006.W	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US03/28073	09 September 2003 (09.09.2003)	09 September 2002 (09.09.2002)

Applicant

WARREN PHARMACEUTICALS, INC.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Mail Stop PCT, Attn: IPEA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Holly Schnizer Telephone No. (703 308-0196

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
JOHN P. MULGREW
SWIDLER BERLIN SHEREFF FRIEDMAN, LLP
3000 K STREET, N.W.
SUITE 300
WASHINGTON, DC 20007

WRITTEN OPINION

(PCT Rule 66)

Date of Mailing *(day/month/year)*	09 SEP 2004
Applicant's or agent's file reference: 20528.0006.W	REPLY DUE: within 1 months/days from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US03/28073	09 September 2003 (09.09.2003)	09 September 2002 (09.09.2002)

International Patent Classification (IPC) or both national classification and IPC

IPC(7): A61K 38/18, 38/16, 38/22; C07K 1/10, 14/505 and US Cl.: 514/8, 12, 814; 530/397, 402, 408, 409, 410, 411

Applicant

WARREN PHARMACEUTICALS, INC.

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

- I ☒ Basis of the opinion
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Rule 66.2 (a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension. See rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 *bis*.
For an informal communication with the examiner, see Rule 66.6

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 09 January 2005 (09.01.2005).

Name and mailing address of the IPEA/US Mail Stop PCT, Attn: IPEA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 872-9306	Authorized officer Valerie Bell-Harrington Holly Schnizer Telephone No. (571) 272-1600

Form PCT/IPEA/408 (cover sheet)(July 1998)

WRITTEN OPINION

International application No.
PCT/US03/28073

I. Basis of the opinion

1. With regard to the elements of the international application:*

☒ the international application as originally filed

☒ the description:
pages 1-49, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the claims:
pages 50-54, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the drawings:
pages 1-1, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☐ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages NONE _____

☐ the claims, Nos. NONE _____

☐ the drawings, sheets/~~fig~~ NONE _____

5. ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed."*

WRITTEN OPINION

International application No.
PCT/US03/28073

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-27	YES
	Claims NONE	NO
Inventive Step (IS)	Claims 1-27	YES
	Claims NONE	NO
Industrial Applicability (IA)	Claims 1-27	YES
	Claims NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-27 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest a man-made erythropoietin product comprising the claimed modifications to the oligosaccharide chains wherein the erythropoietin has a longer serum half-life and tissue protective functionality or methods of using such erythropoietin products. The cited references teach modified EPO proteins with additional glycosylation sites that have increased half-lives. The cited references do not discuss any tissue protective activity of the proteins disclosed therein. In light of the evidence in the description indicating that modified EPO proteins with additional glycosylation sites did not have tissue protective activity, it appears that tissue protective activity would not necessarily be a property of the EPO proteins disclosed in the cited references.

Claims 1-27 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

W. TEN OPINION

International application No.

PCT/ /28073

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claims 1-27 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because the claims are not fully supported by the description. The application, as originally filed, did not describe:the entire genus of erythropoietin molecules that have longer serum half-lives than rhuEPO and tissue protective functionality. The application also does not describe the entire genus of erythropoietin molecules with the claimed modified oligosaccharide chains that would have the claimed activity. A description of a genus may be achieved by means of a recitation of a representative number of species falling within the scope of the genus or a recitation of structural features common to members of the genus, which features constitute a substantial portion of the genus. A generic statement that defines a genus of proteins only by their functional activity does not provide adequate written description. In the case of the claims limited to modifications of oligosaccharide chains, these claims do not provide limitations as to which oligosaccharide chains (including oligosaccharide chains that could be added to the erythropoietin) could be modified to successfully achieve the claimed activities or any limitations on amino acid sequences and the specification does not provide any guidance as to any particular modified EPO that would meet the limitations of the claims. Thus, the genus is enormous and the description does not provide a single species of this genus. Thus, a representative number of species falling within the scope of the genus has not been described and the description is considered to lack adequate written description for the claimed genus.

Claims 1-27 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because the claims are not fully supported by the description. The description does not disclose the claimed invention in a manner sufficiently clear and complete for the claimed invention to be carried out by a person skilled in the art because the description does not teach how to make a man-made erythropoietin product with the claimed activities. The effect of structural changes on protein function especially in vivo is highly unpredictable. In fact, the present description indicates that it was surprising that an EPO analog with an extra glycosylation site at the 32 amino acid, which results in a longer half-live, does not have tissue protective activity (see p. 11 of the Description). The Specification does not provide any guidance as to what modifications can be made to EPO oligosaccharide chains that would successfully provide the claimed acitivities and has not even provided a single example of an EPO modified at the oligosaccharide chains that has the activities as claimed. Therefore, due to the unpredictability of making modified EPO proteins that would have the claimed activities, the lack of guidance or examples of modifications that would successfully result in the claimed activities, the lack of any teachings in the prior art as to how to make the molecules of the claimed genus, it would require undue experimentation to make the modified EPO proteins of the claims. If follows that if the description has not sufficiently taught how to make the products that the methods of using the products also lack enablement.

WRITTEN OPINION

International application No.
PCT/US03/28073

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

TIME LIMIT:
The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.